Analysis of the Human Plasma Proteome with Free-Flow Electrophoresis and LC-MS/MS Technologies

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Objective

Identification of low abundant plasma proteins using free-flow electrophoresis as initial separation step

Introduction

The comprehensive analysis of the human plasma proteome has long been recognized as a challenging analytical task. Human plasma contains the highest protein amount upon human proteome compared with any other sample and the dynamic range of the different proteins is very high[1]. The most abundant proteins are Human Serum Albumin (HSA) and IgG, which together represent 60 – 80% of total protein content[2]. In contrast, proteins like somatomedin B and retinol binding protein are 10^3-fold lower in plasma and interleukin 6 and troponin T are even 10^9-10^{10}-fold lower abundant in plasma[3]. This high dynamic range of protein abundance is a limiting factor in proteome research. In the past, two-dimensional gel electrophoresis (2-DE) has been applied to the characterization of the human plasma proteome. So far, the combination of 2-DE and MS reveals 325 unique proteins[3]. However, this method includes some disadvantages, such as limited loading capacity and dynamic range. To overcome the limitations of the 2-DE approach, two- or multi-dimensional chromatography methods have been developed in recent years. In most cases, the proteins are digested and the generated peptides are separated in different fractions with strong cation exchange chromatography as a first dimension and reversed phase chromatography of the different fractions as a second dimension prior to MS/MS analysis. Using this approach, including very high-pressure reversed phase chromatography, more than 800 proteins could be identified[4,5]. Further strategies include a depletion to remove HSA or HSA and IgG`s from the plasma prior to MS/MS analysis. This should avoid the masking of low abundance proteins through HSA or IgG and therefore make those proteins accessible for MS/MS analysis. However, the different depletion methods have some disadvantages like low loading capacity, lack of specifity[2] or even co-depletion of low abundance proteins bound to HSA.

Therefore, the focus of our study was the improvement of protein separation using Free-Flow Electrophoresis (FFE), a technique that uses isoelectric focusing (IEF) to fractionate proteins according to their pI and that allows a high range of sample amount to be loaded. This pre-fractionation enabled us in combination with LC-MS/MS techniques to identify a high range of proteins.

Methods

Human plasma was fractionated on a FFE using an 8 M urea denaturing protocol. The necessary pH gradient was established using ProLyte reagents. The human plasma was diluted 1:10 with one of the separation media to establish the correct pH and conductivity in the sample. Separated proteins were collected in a 96-well plate. Aliquots of the different protein fractions were directly reduced with DDT, alkylated with iodoacetamide and enzymatical digested with LysC and trypsin.
The generated peptides were analyzed with LC-MS/MS methods using an LTQ-FT or an QTOF2 mass spectrometer. A Mascot search engine was used for database search to identify analyzed proteins and parsed using DBParser.[6]

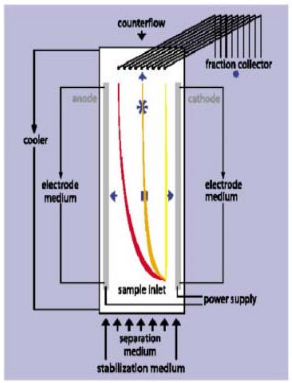

Fig. 1: Schematic scheme of the FFE separation system

- Sample fractions are collected
- Sample flows through the chamber driven by the laminar flow
- Sample is separated in the pH gradient by the electric field

Results

Fig. 2 depicts the employed strategy: Proteins were separated into a 96-well plate according to their pI and enriched in these fractions. The pH difference from one fraction to the next fraction was between 0.1 – 0.2 and the pH gradient in the analyzed fractions ranged from pH 3.8 (fraction 2D) to pH 10.7 (fraction 11E).
Direct double digestion of the fractions with Lys-C and trypsin was possible and the obtained peptides were subsequently analyzed with either QTOF2 or LTQ-FT LC-MS/MS systems.

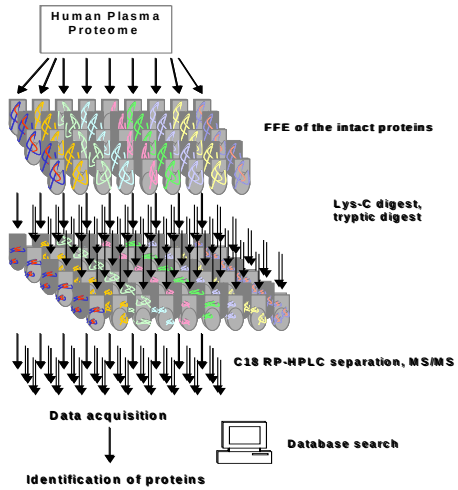

Fig. 2: Strategy to identify human plasma proteome using FFE and LC-MS/MS

Raw data were analyzed with a Mascot search engine, initially using the SWISSPROT_TREMBL database and the obtained results were further classified with DBParser[6]. This analysis led to the identification of a maximum sum of 175 proteins for the QTOF2 and 372 proteins for the LTQ FT, respectively. A detailed comparison of these results showed that 170 proteins were found with both instruments, 5 proteins could only be detected with the QTOF2, whereas 198 unique proteins were only identified with the LTQ FT. This result indicates the much higher sensitivity of the LTQ FT setup when compared to the QTOF2.

Total number of proteins identified using FFE and LC-MS/MS technologies

	QTOF	LTQ-FT
Total number of distinct proteins	5	23
Total number of differentiable proteins	2	15
Total number of equivalent proteins	73	112
Total number of non-redundant equivalent proteins	11	34
Total number of subsumable proteins	6	8
Total number of subset proteins	89	212
Max sum of proteins	175	372

Fig. 3: Total number of proteins identified using FFE and LC-MS/MS technologies; classification according to DBParser[6]

A comparison between our results and an approach using depletion kits to remove high abundant proteins prior to LC-FTICR MS[7] showed that we were able to recognize many of the proteins that were otherwise only accessible for identification after removal of HSA or even HSA and IgG. This result indicates the high potential of the FFE approach for the comprehensive analysis of complex mixtures such as plasma. The pre-fractionation enabled a good separation of plasma proteins in many different fractions. The subsequent analysis of the LC-MS/MS chromatograms detected thus more proteins than the HSA or HSA/IgG depletion approaches (Table 1)[7].

Protein	Native Plasma	HSA-depletion kit	HSA/IgG removal kit	Native Plasma FFE separated
Alpha-1-anitchymotrypsin				
Alpha-1 antitrypsin				
Alpha-2-macroglubulin				
Antithrombin				
Apolipoprotein A-I				
Apolipoprotein A-II				
Apolipoprotein A-IV				
Apolipoprotein C-III				
Apolipoprotein D				
Beta-1B-glycoprotein				
Ceruloplasmin ferroxidase				
Complement component 1q				
Complement component 3				
Complement component 4				
Complement component 8				
Complement factor B				
Cystatin 8				
Cystatin S				
Fibrinogen				
Haptoglobulin				
HSA				
IG J chain				
Ig mu				
Immunolgobulin G				
Inter-alpha-trypsin inhibitor				
Transferrin				
Transthyretin				
Vitamin –D-binding protein				

found
sometimes found
not found

Table 1: Comparison of our FFE-LC-MS/MS approach with an approach using depletion kits before plasma separation and analysis[7]

Conclusions

Using FFE, high abundance proteins were separated from the lower abundance proteins increasing the chance to successfully characterize low abundance proteins. Overall, FFE combined with advanced mass spectrometry technologies can be viewed as a valuable methodology to identify biomarkers from human plasma proteome. Further optimization of FFE pre-fractionation and optimizing the LC-MS/MS parameters should enable use to identify even more low-abundant plasma proteins.

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